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Concentration and origin of lead (Pb) in liver and bone of Eurasian buzzards (*Buteo buteo*) in the United Kingdom

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+This paper is dedicated to the memory of Richard F. Shore

[1]Deceased

ABSTRACT

Ingestion of lead (Pb) derived from ammunition used in the hunting of game animals is recognised to be a significant potential source of Pb exposure of wild birds, including birds of prey. However, there are only limited data for birds of prey in Europe regarding tissue concentrations and origins of Pb. Eurasian buzzards (*Buteo buteo*) found dead in the United Kingdom during an 11-year period were collected and the concentrations of Pb in the liver and femur were measured. Concentrations in the liver consistent with acute exposure to Pb were found in 2.7% of birds and concentration in the femur consistent with exposure to lethal levels were found in 4.0% of individuals. Pb concentration in the femur showed no evidence of consistent variation among or within years, but was greater for old than for young birds. The Pb concentration in the liver showed no effect of the birds' age, but varied markedly among years and showed a consistent tendency to increase substantially within years throughout the UK hunting season for gamebirds. The resemblance of the stable isotope composition of Pb from buzzard livers to that of Pb from the types of shotgun ammunition most widely-used in the UK increased markedly with increasing Pb concentration in the liver. Stable isotope results were consistent with 57% of the mass of Pb in livers of all of the buzzards sampled being derived from shotgun pellets, with this proportion being 89% for the birds with concentrations indicating acute exposure to Pb. Hence, most of the Pb acquired by Eurasian buzzards which have liver concentrations likely to be associated with lethal and sublethal effects is probably obtained when they prey upon or scavenge gamebirds and mammals shot using Pb shotgun pellets.

Capsule: Several characteristics of lead (Pb) contamination of Eurasian buzzards in the United Kingdom are consistent with ingested Pb gunshot being a principal source pathway.

Keywords: stable isotope; shotgun; spent lead ammunition; acute exposure; shooting seasons

1. Introduction

Lead (Pb) is toxic to vertebrates and has adverse effects on most body systems (EFSA 2010). Wild birds are exposed to environmental Pb from several sources, including that occurring naturally in soil and water, emitted from smelters, residues from leaded petrol and paint, lost or discarded fishing weights and spent ammunition (Franson & Pain 2011; Grade et al. 2018; Pain, Mateo & Green 2019). Current exposure of wild animals to Pb derives partly from residues remaining from historical activities, but because anthropogenic emissions have been reduced substantially by recent regulation (EFSA 2010), ammunition is now a frequent source of Pb exposure of birds (see recent review by Pain, Mateo & Green (2019)). Some species, such as gamebirds and waterfowl, mistake spent shotgun pellets deposited during hunting on soil or in wetlands for food items or grit. The frequency of pellet ingestion varies considerably among species, especially waterfowl, and Pb poisoning causes high mortality in some species (Mateo 2009; Green & Pain 2016). Scavenging and predatory birds can be poisoned when lead shotgun pellets and fragments of shot or lead-based bullets embedded in tissue are ingested after they kill or scavenge from shot game animals (Finkelstein et al. 2012; Pain, Mateo & Green 2019). While studies from North America and Europe indicate that a proportion of predatory and scavenging birds die from Pb poisoning (Pain, Mateo & Green 2019), there have been few studies of Pb exposure of these taxa in the UK (Pain & Green 2015).

The concentration of Pb in the bones of wild birds is usually regarded as the best indicator of exposure over the lifetime of the bird, because Pb accumulates in bone and, once deposited, relatively little of it is remobilised (Scheuhammer 1987, Franson & Pain 2011, Krone 2018), although female birds remobilise some Pb from the skeleton when they form eggshells (Finley & Dieter 1978). Because Pb is rapidly excreted and transferred to bone from the blood and soft tissues, its concentration in bone is a less useful indicator of recent exposure and absorption than that in soft tissues, such as blood and liver (Franson & Pain 2011). The half-life of Pb in blood in California condors (*Gymnogyps californianus*) is 14 – 17 days (Green et al. 2008; Fry et al. 2009). Reliable estimates of the half-life of Pb in the soft tissues of other birds of prey are not available, so it is uncertain how much it may vary among species, but the half-life of Pb in soft tissues of vertebrates is generally short compared with that for bone (Agency for Toxic Substances and Disease Registry 2020). Because of the large difference between bone and liver in the lability and accumulation of Pb, we would expect only a weak correlation between bone and liver Pb concentrations across sampled individuals unless there was substantial variation among individuals in their long-term exposure to Pb. Such variation might arise from geographical variation of differences among individuals in behaviour and diet.

In this paper, we analyse data on Pb concentration and isotopic composition in liver and Pb concentration in bones of Eurasian buzzards (*Buteo buteo*) in the United Kingdom (UK) to test several hypotheses, including that ingestion of Pb from ammunition makes a significant contribution to the Pb exposure of this species. The Eurasian buzzard is a widely-distributed bird of prey (Accipitridae) which breeds in much of Eurasia and has been suggested as a suitable sentinel species for assessing the risks to birds of prey from Pb contamination in Europe (Badry et al. 2020). In the UK, buzzards prey upon and scavenge from carcasses of bird and mammal species including lagomorphs (Leporidae), voles (Cricetidae), gamebirds (Phasianidae and Tetraonidae), pigeons (Columbidae) and shorebirds (Scolopacidae and Charadriidae) (Graham, Redpath & Thirgood 1995; Francksen et al. 2016; 2017). Some of the species fed upon by buzzards, particularly lagomorphs, pigeons and gamebirds, are the quarry of hunters and farmers, who predominantly use Pb shotgun ammunition to shoot them (Pain *et al.* 2010). Shotgun pellets and bullet fragments are frequently present in the bodies of unrecovered animals that were shot and killed (Krone 2018), viscera discarded by hunters (Knott et al. 2010) and live animals that are struck but not killed (Tavecchia et al. 2001, Pain et al. 2015). Hence, by feeding on carrion and preying upon these animals, Eurasian buzzards are potentially exposed to dietary Pb from ammunition to a variable extent, depending on the local type and intensity of shooting and the composition of their diet. Additional non-ammunition sources of exposure also exist, as described above.

We performed quantitative assessments of the following six hypotheses. (1) The mean concentration of Pb in liver is lower than that for bone and more variable among individuals because liver concentrations reflect fluctuations in recent exposure to environmental Pb. (2) The mean concentration of Pb in bone is higher for older than younger buzzards, because Pb accumulates in bone over the bird's lifetime, but there is no age dependency for liver Pb, which reflects recent short-term exposure. (3) There is greater within-year and among-year variation in the concentration of Pb in buzzard liver than for bone because the composition of the diet of buzzards is known to vary spatially and temporally as a result of variation in the abundance of preferred food items (Graham, Redpath & Thirgood 1995; Francksen et al. 2016; 2017). (4) Liver Pb concentration is positively correlated with bone Pb concentration across individuals if there is spatial variation and/or consistent individual differences in exposure of buzzards to Pb. (5) If ingestion by buzzards of projectiles or fragments thereof derived from lead-based bullets and lead shotgun pellets is a substantial pathway of Pb exposure relative to other pathways, there will be a consistent pattern of within-year variation in liver Pb concentrations because of the greater level of shooting of game animals in the UK in autumn and winter than in spring and summer. There should not be such variation for bone Pb because its concentration does not reflect short-term exposure. (6) If lead ammunition in the diet of buzzards is a substantial pathway of Pb exposure, relative to other pathways, isotope ratios of Pb from the liver of some individuals should resemble those from widely-used UK shotgun ammunition types, and this resemblance will be strongest in birds with the highest liver Pb concentrations.

2. Materials and methods

2.1 Buzzard sample collection and preparation

Specimens ($n = 220$) were obtained of Eurasian buzzards found dead or dying in the wild in the United Kingdom in the period 2007–2018. Requests were made to the public, birdwatchers and wildlife managers through bird journals, newsletters and other communications, for bodies of birds of prey found dead. Carcasses were sent to the UK Predatory Bird Monitoring Scheme (PBMS) of the Centre for Ecology and Hydrology and to the Raptor Health Scotland project at the Royal (Dick) School of Veterinary Studies (University of Edinburgh). In addition, carcasses were handed in to staff at the International Centre for Birds of Prey and the Royal Society for the Protection of Birds. Carcasses were obtained opportunistically and causes of death were uncertain in many cases and might not have been representative of those for the population at large. Collection localities were widely scattered across Britain, but with only one specimen from Northern Ireland (Supplementary Fig. S1). The day of collection was reported for 65% of carcasses and the calendar month within which collection occurred was reported for 99%. We therefore took the midpoint of the month of collection for all samples as the date used in our analyses of variation over time.

Carcasses were stored deep-frozen at -20°C and examined in batches. The approximate age was determined from plumage characteristics (Baker 2016). Birds were assigned to Euring age classes (EURING 2010), but the degree to which this was possible varied considerably among specimens. For the purposes of the present analysis we placed specimens into two classes: young birds collected in the calendar year of hatching (Euring class 3) and birds older than this (Euring class 4). After thawing, a sample of liver was excised and stored in a plastic vial. A femur and, in a few cases, also a humerus, was dissected out, and as much soft tissue as possible trimmed off. Comparison of the Pb concentration in the humerus with that in the femur of the same bird showed that the two were similar and highly correlated (Supplementary Material and Supplementary Fig. S2), so only femur Pb values were used in the analysis. The bone was placed in a plastic zip-lock bag and re-frozen at -20°C to await further processing and analysis. Bone samples were further prepared by placing them into containers with dermestid beetle larvae, which consumed almost all of the remaining adherent soft tissue.

2.2 Determination of Pb concentrations in livers and bone

Protocols for the determination of Pb concentrations in buzzard tissues are given in the Supplementary Material. We have expressed concentrations throughout as μg kg^{-1} d.w., which is equivalent to parts per billion. Our results can be converted to mg kg^{-1} d.w. and parts per million by dividing them by 1000.

2.3 Biological significance of tissue concentrations of Pb

Several proposals have been made concerning the biological significance of Pb concentrations in the tissues of birds of prey. We followed Pain, Sears & Newton (1995) in considering that a liver Pb concentration in excess of 6000 μg kg^{-1} d.w. (~2000 μg kg^{-1} w.w.) is likely to have resulted from abnormally high exposure to Pb, and a concentration exceeding 20000 μg kg^{-1} d.w. (~6000 μg kg^{-1} w.w.) in liver is indicative of acute exposure and is likely to have caused mortality. For bone, we followed Mateo et al. (2003) in regarding a bone Pb concentration in excess of 10000 μg kg^{-1} d.w. as being elevated, and a concentration exceeding 20000 μg kg^{-1} d.w. as being compatible with lethal poisoning.

2.4 Selection and sourcing of shotgun cartridges for Pb isotope analysis

We wished to measure Pb isotope ratios in Pb shotgun pellets taken from brands of shotgun cartridges most widely used in the UK during our study period. To select appropriate brands, we used the results of a survey of a large sample of UK shooters conducted by GunsOnPegs and Strutt & Parker (2017). This survey reported the market share of shotgun cartridges made by 19 manufacturers which had been used by survey respondents in 2017. Five of these 19 manufacturers sold 90% of all cartridges. We obtained cartridges, suitable for use in 12-gauge shotguns, made by these five manufacturers (Gamebore, 27% of market share; Hull, 23%; Eley, 22%; Lyalvale, 9%; RC, 9%). In 2017 and 2018, two holders of UK shotgun licences purchased the cartridges from retailers, obtaining

18 boxes of cartridges of 12 types of cartridges containing #5 and #6 size pellets (sizes commonly used for hunting lagomorphs, gamebirds and pigeons). We removed shot from three cartridges from each box of cartridges and mixed them together. We took three pellets from this mixture and digested them together. This comprised one sample. In our comparison (see below) of isotope results from these shotgun cartridges purchased in 2017 - 2018 with isotope results from buzzard liver samples collected over an overlapping but longer period (2008 – 2018), we assumed that the cartridge brands used and the isotopic composition of the Pb in them during the entire buzzard sampling period were similar to those in 2017 - 2018. Ideally, we would have purchased cartridges of widely-used brands in every year of the buzzard sampling period, but this was not done.

2.5 Isotope analysis of Pb shot pellets from shotgun cartridges and Pb in buzzard liver samples

Protocols for the determination of isotope composition of Pb from ammunition cartridges and buzzard liver are given in the Supplementary Material.

2.6 Statistical analysis of the concentration of Pb in tissues

There were six buzzard samples with concentrations of Pb below the LOD (100 μg kg^{-1} d.w.), all of which were in liver samples. We replaced these values with 0.5 x LOD (here 50 μg kg^{-1} d.w.) for statistical analyses. We transformed concentrations to natural logarithms before analysis. We calculated the mean and standard deviation of $\log_e$-transformed values to model log-normal distributions of concentrations and estimate geometric means, and tested the conformity of the empirical distribution to the fitted log-normal distribution using the Kolmogorov-Smirnov one-sample test (Siegel & Castellan 1988). We used Bartlett's test of homogeneity of variance (Snedecor & Cochran 1991) to test whether the variances of $\log_e$-transformed Pb concentrations were similar in liver and femur. We used the Pearson correlation coefficient for assessments of correlation. When relating concentrations of Pb in the femur, humerus and liver of the same bird to one another in pairwise analyses, we recognised that the variables were all subject to measurement error. Therefore, it would have been incorrect to use simple ordinary least-squares linear regression which assumes that the independent variable has been determined without error. We therefore used reduced major axis regression, which assumes that the errors are equal for the two variables (Sokal & Rohlf 1969). Exact binomial confidence limits (Diem 1962) were calculated for proportions of specimens with concentrations of Pb considered to be of biological significance.

For the analysis of tissue Pb concentrations in relation to collection date (i.e. time elapsed since the beginning of the study period), time within the year (i.e. season) and age class, we used $\log_e$-transformed concentrations as the dependent variable and fitted least squares regression models. We devised a set of seven regression models which included all combinations of the three independent variables. The effect of age class was modelled as a binary factor (hatched in the current calendar year or older). Collection date was the mid-point of the month of collection and was modelled by piecewise regression with breakpoints assumed to occur on the same date in each calendar year. The slopes of the regression lines between each successive pair of breakpoints were estimated separately. In addition, the effect of time of year within calendar year was modelled as a sine function in which the phase and amplitude of the sinusoidal relationship were assumed to be the same in each year. The timing of the annual breakpoint in the modelling of the effect of collection date and also of the phase of the sinusoidal function of the effect of time of year were both estimated using a bisection search algorithm (Kalbfleisch 1985) to determine the values of each that minimised the residual sums of squares. The three effects were assumed to be additive in terms of log-concentrations. Models were fitted using a non-linear least-squares procedure. The performance of models within the set for each tissue was compared by calculating Akaike's Information Criterion adjusted for small sample size (AIC_c) and AIC_c weights for each of the models in the set (Burnham & Anderson 2002). We selected the model with the lowest AIC_c. We summed the AIC_c weights across all the models in the set in which a variable was included to obtain an indication of the relative importance of the three variables (Burnham & Anderson 2002).

Data were available on liver Pb concentration from specimens collected across the whole of our study period, but bone samples were collected and processed over a more restricted period, with all but seven specimens being collected in 2013 – 2015. This restricted sampling precluded the use of the piecewise modelling approach for periods with sparse data. We therefore restricted the analysis of variation in bone Pb concentrations in relation to collection date, time of year and age class to 2012 – 2016. This led us to exclude three values for specimens collected in 2008 – 2011.

2.7 Statistical analysis of Pb isotope ratios

We performed our analysis of Pb isotope ratios as a sequence of three logical steps. Step 1 was to characterise the isotope ratios of Pb pellets from shotgun cartridges of brands widely used in the UK. Step 2 characterised the $^{206}Pb/^{207}Pb$ and $^{208}Pb/^{206}Pb$ isotope ratios for Pb from buzzard liver samples. This required that we model the observed buzzard liver data as comprising values characteristic of shotgun pellets (defined in Step 1), together with others derived from various additional unknown sources. Step 3 assessed the extent to which the probabilities of liver samples being members of the shotgun set and the additional sets were correlated with the concentration of Pb in the liver sample. The procedure for these analyses is set out in detail in the Supplementary Material.

2.8 Estimation of the proportion of the mass of Pb in liver likely to have been derived from shotgun ammunition

We estimated the proportion of the mass of Pb in liver likely to have been derived from shotgun ammunition by multiplying together three quantities for every value of liver Pb concentration in the observed range. These quantities were (1) the probability density of the liver concentration of Pb, (2) the concentration itself, and (3) the proportion of Pb at that concentration estimated from the analysis of isotope ratios to be derived from shotgun pellets. This three-way product was then summed across all concentrations and divided by the sum, across all concentrations, of the two-way product of quantities (1) and (2). This calculation was also performed for two subsets of the liver Pb concentration distribution: the range of concentrations considered to be abnormally high (>6000 μg kg^{-1} d.w.) and the range of liver Pb levels indicative of acute exposure (>20000 μg kg^{-1} d.w.). The selection of these threshold concentrations was explained in section 2.3. Quantity (1) was calculated using the mean and standard deviation of the log-normal distribution of Pb concentrations, fitted as described in section 2.6. Quantity (3) was obtained from the regression model of the logit-transformed proportion of data attributable to the shotgun set in relation to liver Pb concentration (Step 3 of section 2.7). Confidence limits for the proportion of the mass of Pb in liver derived from shotgun ammunition were obtained by a bootstrap method (Manly 2006). The calculations described above were repeated for 10,000 bootstrap samples of liver Pb concentration and isotope data drawn at random, with replacement, from the observed data. The bootstrap estimates were ranked and bounds of the central 9,500 values were taken to be the 95% confidence interval.

3. Results

3.1 Means and distributions of concentrations of Pb in the liver and femur

The arithmetic mean concentration of Pb in buzzard livers was 2573 μg kg^{-1} d.w. (n = 187, standard deviation = 7516 μg kg^{-1}; range- <100 to 85400 μg kg^{-1}). The median concentration was 722 μg kg^{-1} d.w.. The geometric mean concentration was 795 μg kg^{-1} (95% confidence interval 648 to 974 μg kg^{-1}). For the femur, the arithmetic mean concentration was 5460 μg kg^{-1} d.w. (n = 125, standard deviation = 10669 μg kg^{-1}; range- 146 to 110000 μg kg^{-1}). The median concentration was 3240 μg kg^{-1} and the geometric mean concentration was 2951 μg kg^{-1} (95% confidence interval 2440 to 3570 μg kg^{-1}). Hence, the geometric mean concentration of Pb in the femur of Eurasian buzzards was nearly four times higher than, and significantly different from, that for liver (Welch's t-test, t = 9.24, d.f. = 304.6, P <0.0001). The distributions of Pb concentrations in both the liver and the femur were approximately log-normal (Fig. 1). For both tissues, the empirical distribution did not depart significantly from that expected from the fitted log-normal distribution (Kolmogorov-Smirnov one-sample tests: liver, D = 0.037, P > 0.20; femur, D = 0.050, P > 0.20). Log$_e$-transformed concentrations of Pb in samples of liver were significantly more variable than concentrations in the femur (standard deviation of $\log_e$-transformed concentrations for liver SD = 1.42; femur SD = 1.08; Bartlett's test, $\chi^2 = 10.38$, $P = 0.001$).

The proportion of specimens with abnormally high levels of Pb in the liver (>6000 μg kg^{-1} d.w.) was 8.0% (95% confidence interval, 4.6 to 12.9%) and the proportion with liver concentrations indicating acute exposure (>20000 μg kg^{-1} d.w.) was 2.7% (95% confidence interval, 0.9 to 6.1%). The proportion of specimens with elevated Pb concentrations in the femur (>10000 μg kg^{-1} d.w.) was 9.6% (95% confidence interval, 4.7 to 15.7%) and the proportion with femur concentrations compatible with lethal poisoning (>20000 μg kg^{-1} d.w.) was 4.0% (95% confidence interval, 1.3 to 9.3%).

3.2 Relationship of Pb concentration in the femur to that in the liver

There was a highly significant positive correlation between the $\log_e$-transformed Pb concentration in the femur and that in the liver for the 92 individuals for which both measurements were available ($r = 0.394$, $P = 0.0001$; Fig. 2). The relationship between $\log_e$-transformed concentrations in the two tissues was approximately linear, but with substantial scatter. The greater variation among birds in Pb concentration in the liver than in the femur, previously noted in section 3.1, is also evident in Fig. 2. The Pb concentration in the femur was larger than that in the liver of the same individual in 87% of cases (80/92, Sign Test, $z = 7.19$, $P < 0.0001$), but this tendency was least pronounced for individuals with the highest Pb concentrations in the liver, indicative of acute exposure (Fig. 2). The mean concentration of Pb in the femur tended to increase by a smaller proportion for a given proportional increase in the mean liver Pb concentration, which is reflected in the slope of the reduced major axis regression (RMA) of femur Pb on liver Pb (Fig. 2). The RMA slope of $\log_e$ femur Pb concentration relative to $\log_e$ liver Pb concentration was considerably lower (0.753) than the slope of 1 that would occur if femur Pb concentration was directly proportional to liver Pb concentration. The 95% confidence interval of the RMA slope did not overlap the value of 1 (95% confidence interval: 0.610 to 0.896).

3.3 Relationship of Pb concentration in the liver and femur to year, time of year and age class

Concentrations of Pb in liver samples are shown in relation to date of collection in Fig. 3. Regular annual fluctuations in the concentration in the liver are apparent from this graph, with peaks occurring in late winter and troughs in late summer, but there also appear to be differences among calendar years. The regression model with the lowest AIC_c of the set of seven models examined was Model 6, which includes a piecewise effect of collection date combined with a sinusoidal effect of time of year (Table 1). An effect of age class was not supported by these analyses. The relative importance values (Burnham & Anderson 2002) of collection date, sinusoidal effect of time of year and age class were 0.991, 0.999 and 0.232 respectively, which indicates that collection date and the sinusoidal effect of time of year both had strong effects on liver Pb concentration, but that the effect of age class was minor. The fitted sinusoidal term in Model 6 indicated a peak in Pb concentrations on 11 February and a trough on 12 August, with the geometric mean concentration at the peak being 3.9 times the geometric mean concentration at the trough (95% confidence interval of the ratio, 2.2 to 7.0).

No obvious changes in Pb concentration in the femur with collection date or time of year are apparent from a graph (Supplementary Fig. S3). The regression model with the lowest AIC_c of the set of seven models examined was Model 1, which includes only the effect of age class (Table 1). Effects of collection date and a sinusoidal effect of time of year were not supported by regression analyses. The relative importance values of collection date, sinusoidal effect of time of year and age class were 0.226, 0.326 and 0.668 respectively, which indicates that, in marked contrast to the analysis of liver Pb, age class had a much stronger effect on femur Pb concentration than collection date or the sinusoidal effect of time of year. The geometric mean concentration of Pb in the femur samples from buzzards in the calendar year of hatching was about half (1614 μg kg^{-1}) of that of older birds (3242 μg kg^{-1}).

3.4 Isotope ratios of Pb pellets from shotgun cartridges

$^{206}Pb/^{207}Pb$ and $^{208}Pb/^{206}Pb$ isotope ratios for Pb from 18 shotgun cartridges produced by five manufacturers whose cartridges are widely used in the UK are shown in Supplementary Table S1. A biplot of the $^{208}Pb/^{206}Pb$ ratio against the $^{206}Pb/^{207}Pb$ ratio indicated that a bivariate normal distribution gave a reasonable approximation to the data (Fig. 4). Inspection of Fig. 4 suggests that Pb pellets from the same manufacturer had similar isotope ratios to one another and tended to be different from, though sometimes overlapping with, those of other manufacturers. Ideally, we would have analysed larger samples of cartridges from every manufacturer and estimated the bivariate normal parameters for each one. However, we did not process sufficient samples to do this and therefore estimated the bivariate normal parameters for the cartridges of all five manufacturers combined.

3.5 Isotope ratios of Pb from Eurasian buzzard liver samples

A biplot of the $^{208}Pb/^{206}Pb$ ratio against the $^{206}Pb/^{207}Pb$ for samples from 181 Eurasian buzzards shows a much wider scatter of values than the shotgun pellet values and also indicates that a single bivariate normal distribution would not provide a good description of the data (Fig. 5). We therefore fitted a model in which we assumed that the data were derived for a mixture of several sets of samples, each of which had a different bivariate normal distribution pattern. We assumed that the proportion of samples attributed to each set differed among the sets. We fitted different versions of the model, all of which included the shotgun set with bivariate normal parameters defined above. We also assumed that there were between one and five additional sets, with unknown parameter values estimated from the data. The proportions of samples in each set were also estimated. Comparison of AIC_c values from models with different numbers of additional sets showed that the model with three additional sets gave the lowest AIC_c and was therefore best supported by the data (Supplementary Table S2). Bivariate normal 95% ellipses for most of the sets defined by this model overlapped with each other substantially (Fig. 5), though the Set 1 ellipse did so only marginally. The ellipses for Sets 2 and 3 overlapped with each other and also with the shotgun pellet set.

3.6 Similarity between isotope ratios of Pb fromEurasian buzzard liver samples and shotgun pellets in relation to the concentration of Pb in the liver

There was a significant positive correlation, across progressively increasing deciles of Pb concentration, between the logit-transformed proportion of liver samples within a decile attributed to the shotgun set and the mean of the $\log_e$-transformed Pb concentrations of the samples in that decile (Fig. 6; r = 0.701, t_8 = 2.78, P = 0.024). None of the equivalent correlations for the three additional sets approached statistical significance (Set 1; r = 0.006, P = 0.986; Set 2; r = -0.172, P = 0.635; Set 3; r = 0.514, P = 0.128). We conclude that the isotope ratios of buzzard liver samples with high Pb concentrations resembled those of Pb shotgun pellets much more closely than did samples with low concentrations. The fitted regression (Fig. 6) suggests that much of the Pb in the livers of buzzards with the highest observed concentrations was derived from Pb shotgun pellets.

3.7 Proportion of the mass of Pb in liver likely to be derived from shotgun ammunition

The estimated proportion of the mass of Pb in the liver of all sampled buzzards that was attributable to widely-used types of shotgun pellets was 57% (95% confidence interval; 30 – 73%). The equivalent proportion for the part of the distribution of Pb liver concentration considered to indicate abnormally high Pb levels (>6000 μg kg^{-1} d.w.) was 77% (95% confidence interval; 44 – 95%) and that for the part of the distribution considered to indicate acute exposure (>20000 μg kg^{-1} d.w.) was 89% (95% confidence interval; 57 – 99%).

4. Discussion

The concentrations of Pb we found in livers of 187 Eurasian buzzards collected between 2007-2018 were broadly similar to those determined for a smaller sample (n = 56) of buzzards found dead in the UK in 1981 – 1992 (Pain, Sears & Newton 1995). The proportions of birds with levels of Pb indicating elevated or acute exposure were broadly similar and not significantly different between the earlier study and ours. In 1981 -1992, liver concentration exceeded 6000 μg kg^{-1} d.w. for 5.3% of birds (cf. 8.0% in our sample) and exceeded 20000 μg kg^{-1} d.w. for 1.8% of birds (cf. 2.7% in our sample) (two-tailed Fisher exact tests, P = 0.581 and P = 1.000 respectively). A systematic review by Monclús, Shore & Krone (2020) reported arithmetic mean Pb concentrations in liver samples from Eurasian buzzards collected in five European countries (France, Italy, Poland, Portugal and Spain). To this we added results for buzzards from Denmark, which were reported by Kanstrup *et al.* (2019) after the systematic review had concluded. We followed Monclús, Shore & Krone (2020) in multiplying the mean value of Kanstrup *et al.* by 3.1 to convert it from per unit wet weight to per unit dry weight. Comparing the results for the UK with those for the other six countries, we found that the mean concentration in liver in the UK was exceeded only by that for Italy. Monclús, Shore & Krone (2020) also reported arithmetic mean Pb concentrations in bone from buzzards collected in four European countries (Italy, Netherlands, Poland and Spain). The mean concentration for UK buzzards lay in the middle of this distribution, being exceeded by the means for the Netherlands and Poland.

Our study and that of Pain, Sears & Newton (1995) both suggest that exposure to Pb may have caused some buzzard deaths in the UK, but the proportion cannot be estimated reliably. Exposure to Pb may increase the risk of death in birds of prey indirectly, by causing changes in behaviour and physiology, even at levels well below those expected to cause acute toxicity. In GPS-tagged golden eagles (*Aquila chrysaetos*) in Sweden, mean flight height and mean movement rate were both approximately halved when Pb concentration in the blood exceeded thresholds of 17 and 25 μg kg^{-1} w.w. (1.7-2.5 μg dL^{-1}) which is well below accepted thresholds for both subclinical and lethal effects (Ecke et al. 2017). It is possible that sub-lethal exposure to Pb may increase the risk of death by causing such changes in behaviour. Effects of exposure to Pb on flight behaviour might result in a higher rate of accidental death through collisions with man-made structures. Kelly & Kelly (2005) determined blood levels of Pb in mute swans (*Cygnus olor*) admitted to a wildlife rehabilitation centre with injuries, diseases or Pb poisoning. The proportion of birds admitted because of collisions with overhead cables was highest for birds with moderately elevated concentrations of Pb in the blood. It was hypothesised that swans with low and moderate blood Pb concentrations flew with normal frequency, but that those with moderate Pb levels were less able to avoid obstacles. Swans with higher than moderate blood Pb were suggested to suffer sub-lethal effects which made them unlikely to fly and they were therefore unlikely to collide with structures. Regarding possible physiological effects, previous studies have detected an adverse effect of Pb on ALAD activity in birds at blood Pb levels below 20 μg dL^{-1}, and as low as 3 μg dL^{-1} (Finkelstein et al. 2012, Martinez-Haro et al. 2011, Espín et al. 2015, Newth et al. 2016, Herring et al. 2020).

We expected the concentration of Pb in bone to be larger on average and less variable among individuals than the concentration in liver. Both of these expectations are supported by our results. We also expected that the concentration of Pb in bone would be larger for older than for younger buzzards, because it accumulates over the bird's lifetime, but we did not expect a similar difference for liver Pb because its concentration reflects recent short-term exposure. As expected, we found that the geometric mean concentration of Pb in the femur of buzzards hatched and collected in the same calendar year was about half of that for older birds, but that there was no significant effect of age class on liver Pb.

We expected there would be substantial variation over time in the concentration of Pb in the livers of Eurasian buzzards, but much less temporal variation for bone Pb. Our analyses support this expectation, indicating large differences among years, for liver Pb but not for femur Pb. The reasons for these differences between years are not known, but they are most likely driven by dietary preferences and fluctuations in the availability of preferred foods. The diet of buzzards is known to vary spatially (Graham, Redpath & Thirgood 1995; Francksen et al. 2016; 2017) and the abundance of some of their principal prey species, such as rabbit (*Oryctolagus cuniculus*) and field vole (*Microtus agrestis*) also varies substantially among years (Trout & Tittensor 1989; Village 1990; Lambin, Petty & Mackinnon 2000). Differences among years in the locations from which dead birds were collected might also contribute to this apparent variation among years, but assessment of this possibility requires a sophisticated spatio-temporal analysis of our data, which is beyond the scope of our present study.

We expected that the degree to which femur and liver Pb concentrations would be positively correlated across sampled individuals would depend upon the amount of variation among individual buzzards in their long-term exposure to Pb. Our finding of a highly significant positive correlation is consistent with there being substantial and consistent variation among individuals in exposure to Pb. This might be due to geographical variation in exposure or to individual differences in behaviour or diet, or both.

Studies of scavenging raptors in Europe and the USA (reviewed in Pain & Green 2015) show that both levels of shot ingestion (presence of shot in regurgitated pellets) and blood Pb concentrations peak during the hunting season. If Eurasian buzzards are exposed to lead ammunition when they feed on tissue from scavenged animals killed by shooting or wounded prey animals, we would expect that the concentration of Pb in the liver would increase within the shooting season and decline outside it. Although non-Pb bullets and shotgun cartridges are available in the UK, most animals shot for sport or for pest control are killed using lead ammunition. Pain et al. (2010) found that Pb shot had been used to kill 91% of five species of terrestrial gamebirds and mallard (*Anas platyrhynchos*) purchased from

UK retailers for which they determined the metallic composition of shotgun pellets recovered from the birds' bodies. The use of lead bullets and lead shotgun pellets is legal for most shooting in the UK, although the shooting of wildfowl, coot (*Fulica atra*) and moorhen (*Gallinula chloropus*) and/or over certain or all wetlands with lead shotgun pellets has been banned. Details of the regulations vary among UK countries (Stroud 2015). However, compliance with the regulation that applies to England has been poor (ca. 30%) throughout the period since it came into effect (Cromie et al. 2015).

Buzzards scavenge and prey upon both birds and mammals. Of animals shot for sport in the UK, 95% are birds and 5% are mammals (Public and Corporate Economic Consultants 2006), so the shooting seasons for birds are likely to have the largest influence on variation within years in the exposure of buzzards to Pb from ammunition. Although legal shooting seasons for birds vary slightly among the four UK countries, they are approximately October to January for common pheasant (*Phasianus colchicus*), September to January for partridges (*Perdix perdix* and *Alectoris rufa*) and for ducks and geese (Anatidae), and 12 August to 10 December for red grouse (*Lagopus lagopus*). Shooting of common woodpigeons (*Columba palumbus*) occurs throughout the year, but is most frequent in winter, often in response to woodpigeons grazing autumn-sown farm crops. Pheasants and partridges together comprise 83% of the 21 million birds of all species shot annually in the UK (Aebischer 2017), so it is the timing of their shooting seasons that is likely to be most relevant here. Hence, our finding of an increase from August to February in the concentration of Pb in the livers of buzzards is consistent with a probable increase over the shooting season in the availability to buzzards of carcasses of unrecovered shot birds and birds that died from other causes with embedded or ingested shot in their bodies. While crippling of pheasants not killed immediately by shooting are considered to be an important cause of mortality, such events are self-reported by hunters and we could find no reliable estimates for the UK. In the USA, crippling as a percentage of male pheasants shot and retrieved are usually in the range 10-30% (Edwards 1988; Kania & Stewart 2009). The prevalence of embedded shot in wild-trapped ducks in the UK in the 1980s was 15-27% (Pain et al. 2015). The prevalence of ingested shot in pheasants in the UK is probably lower than for embedded shot. A UK study found a 3% incidence of ingested shot in the gizzards of 437 pheasants from 22 shooting estates (Butler *et al.* 2005). Higher levels have been reported from some studies in the USA (e.g. 23% and 35%, Dutton & Bolen 2000; Kreager *et al.* 2008). Bone Pb concentration represents long-term exposure to environmental Pb, so we did not expect or observe a consistent annual pattern in femur Pb concentration.

Eurasian buzzards frequently scavenge from the carcasses of animals killed by collisions with road traffic. Surveys along roads in the UK found that 38% of road-killed birds overall were pheasants, but this proportion was much higher (50-70%) from October to April than in June to August (ca. 10%) (Madden & Perkins 2017). This seasonal pattern in the proportion of road-killed birds that are pheasants resembles the sinusoidal annual cycle in the concentration of Pb in the livers of buzzards, suggesting that road-killed pheasants with embedded or ingested shot are a possible source of Pb contamination for scavenging buzzards.

Our analysis of isotope ratios of Pb in Eurasian buzzard livers, indicates that much of it is from Pb shotgun pellets, but that some comes from a range of other background sources, probably including environmental pollution and underlying geology. Pb acquired by buzzards in the UK from lead ammunition is probably ingested episodically, but in concentrated amounts. When that occurs, ammunition-derived Pb will outweigh the background Pb isotope signature from other sources in liver and other soft tissues which have labile Pb. By contrast, non-ammunition background Pb is likely to be acquired as a mixture from multiple diffuse sources. Hence, it is probably not feasible to clearly identify the origins of the Pb not derived from shotgun pellets by comparing the parameters of the three non-shotgun bivariate normal distributions identified by our analysis of buzzard isotope ratios with published isotopic characteristics of background environmental Pb from individual non-ammunition sources. Detailed data on the spatial patterns of exposure to the various different potential background sources of Pb and their isotopic composition in the UK are currently insufficient for attribution of background Pb to particular sources and exposure pathways.

If Eurasian buzzards are exposed to substantial amounts of dietary Pb when they feed on tissue from animals killed or wounded by lead ammunition, we would expect that the degree of resemblance between isotope ratios of Pb from the liver and those from widely-used types of ammunition would be positively correlated with liver Pb concentration. We suggested earlier that ammunition used to kill birds is likely to be a much larger source of ammunition-derived Pb for buzzards than that used to kill mammals. The great majority of birds shot in the UK are killed using shotgun pellets, so we expected the isotope ratios of Pb from buzzard livers to resemble ratios for pellets from widely-used cartridge brands more closely as liver Pb concentration increased. We found that the similarity of liver isotope ratios to those of shotgun pellets increased strongly with liver Pb concentration. The buzzards with the highest liver Pb concentrations had isotope ratios consistent with most of the Pb being derived from ammunition. This finding is in accord with conclusions drawn from many other studies of Pb exposure of predatory and scavenging birds around the world (Pain, Mateo & Green 2019), but is unusual in making an estimate of the proportion of liver Pb derived from shotgun ammunition, which was more than half in all buzzards sampled and 89% in the birds with liver Pb concentrations indicating acute exposure.

We found differences in isotope characteristics among different brands of cartridges that we analysed, which were purchased in 2017-2018. This suggests that the sources of recycled Pb or ores, which vary in isotopic characteristics (Sangster, Outridge & Davies 2000), differed among brands and might also change over time. For shotgun pellets recovered from regurgitated pellets of red kites (*Milvus milvus*) collected in the winter of 2003 from one roost site in England, Pain et al. (2007) found that $^{206}Pb/^{207}Pb$ and $^{208}Pb/^{206}Pb$ isotope ratios of 73% of their sample of 11 pellets lay outside the 95% ellipse of the bivariate normal distribution we fitted to our data on pellets from cartridges purchased in 2017 and 2018. This difference might be due to the small sample, which might have been from scavenged animals killed by just one hunter. However, it is also possible that the principal sources of Pb used to manufacture shotgun pellets, and hence their isotopic characteristics, may have changed during the 14 years between the two studies. Published comparisons of Pb isotope ratios between ammunition and wildlife samples often do not check that the types of ammunition analysed are representative of those used at the times and places where the wildlife samples were obtained. We recommend that care is taken in future studies to obtain as good a match as possible.

5. Conclusions

Concentrations of Pb consistent with acute exposure were found in the livers of 2.7% of Eurasian buzzards and Pb concentrations in the femur consistent with exposure to lethal levels were found in 4.0% of birds. Pb concentration in the femur did not vary consistently among or within years, but the concentration in old buzzards was about twice that for young birds. For Pb concentration in the liver, there was no effect of the birds' age, but marked variation among years and a consistent tendency for concentration to increase substantially within years during the UK gamebird hunting season. The stable isotope composition of Pb from buzzard livers resembled that of Pb from the types of shotgun ammunition widely-used in the UK most strongly for birds with a high Pb concentration in the liver. Stable isotope results suggested that 57% of the mass of Pb in livers of all of the buzzards sampled was derived from shotgun pellets, with this proportion being 89% for the birds with concentrations indicating acute exposure to Pb. Pb isotope ratios from different commercial brands of shotgun cartridges varied, so it is important to compare results from representative brands with those from wildlife samples.

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This paper is dedicated to the memory of our co-author and friend Professor Richard Shore (Centre for Ecology and Hydrology). Richard was central to the completion of this study, and as always, worked collaboratively and supportively towards its realisation. Those fortunate enough to have worked alongside Richard will miss him and his contributions to our field. We are honoured to have worked with him.

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Table 1. Comparison of the performance of seven regression models of the concentration of Pb in samples of liver (n = 179) and of bone from the femur (n = 118) of Eurasian buzzards in the UK. Models differed according to which of the three independent variables (age class, collection date and phase of the annual cycle) were included (Y) or excluded (N), as indicated in the Model specification columns. For each model, the number of fitted parameters (NP), ΔAIC_c (the difference in AIC_c between the model and that with the lowest AIC_c of the set) and the AIC_c weight are given. The model with the lowest AIC_c is shown in bold for each tissue.

	Model specification			Liver			Femur		
Model code	Age class	Collection date	Annual cycle	NP	ΔAIC_c	AIC_c wt	NP	ΔAIC_c	AIC_c wt
0	N	N	N	1	25.1	<0.001	1	2.07	0.131
1	Y	N	N	2	24.8	<0.001	**2**	**0.00**	**0.368**
2	N	Y	N	14	16.5	<0.001	7	3.47	0.065
3	N	N	Y	3	9.7	0.006	3	2.47	0.107
4	Y	Y	N	15	13.3	0.001	8	2.41	0.110
5	Y	N	Y	4	11.3	0.003	4	1.56	0.168
6	N	Y	Y	**16**	**0.0**	**0.762**	9	5.09	0.029
7	Y	Y	Y	17	2.4	0.229	10	5.65	0.022

LEGENDS TO FIGURES

Fig. 1. Exceedance (negative cumulative) distributions (stepped lines) of the concentration of Pb (μg kg^{-1} d.w.) in samples of (a) liver (n = 187); and (b) bone from the femur (n = 125) of Eurasian buzzards. The curves show fitted log-normal distributions. The long-dashed vertical lines show concentrations considered to result from abnormally high exposure (a) or elevated levels (b) and the short-dashed lines denote acute exposure and absorption (a) or compatibility with lethal poisoning (b) (see text).

Fig. 2. Concentration of Pb (μg kg^{-1} d.w.) in samples of bone from the femur in relation to that in the liver for 92 Eurasian buzzards. The solid line shows the reduced major axis regression $\log_e$(Femur) = 2.924 + 0.753 $\log_e$(Liver).

Fig. 3. Concentration of Pb in the liver for Eurasian buzzards in the UK in 2007 - 2018 in relation to collection date. Each symbol represents a determination from one individual. Modelled values (curve) are from the model with the lowest AIC$_c$ (Model 6) of the set of models presented in Table 1. This model includes a piecewise regression effect of collection date and a sinusoidal effect of time of year, with peaks in February and troughs in August. Results for young collected in the calendar year of hatching (triangles) and older birds (circles) are distinguished, but there was no significant effect of age class on Pb concentration in the liver. Vertical grey lines show calendar years.

Fig. 4. Isotope ratio biplot for Pb shotgun pellets from five manufacturers; grey square = Gamebore; black circle = RC, white circle = Eley; grey triangle = Lyalvale; black diamond = Hull. Each point represents a value for pellets from a single box of cartridges. The ^{208}Pb/^{206}Pb ratio is plotted against the ^{206}Pb/^{207}Pb ratio. The bivariate normal ellipse containing 95% of the modelled probability is shown.

Fig. 5. Ellipses containing 95% of the probability from a bivariate normal model of isotope ratios in liver samples from Eurasian buzzards. The ellipse fitted to data for Pb shotgun pellets from cartridge brands widely used in the UK is shown by the thick line and is the same as that in Figure 4. The model also identified three additional sets with ellipses labelled Sets 1-3 and shown by the thin lines. The points represent values for individual buzzards. Individuals with liver Pb concentrations indicative of acute exposure and absorption (>20000 μg kg-1 d.w.) are shown as red circles.

Fig. 6. Proportion of samples of liver from Eurasian buzzards attributed to the set having the characteristics of Pb shotgun pellets from cartridge brands widely used in the UK in relation to the concentration of Pb in the liver. Points represent proportions of samples and mean concentrations calculated separately for each decile (n = 18 or 19 per decile) of the concentration distribution. The curve is the fitted ordinary least squares regression of logit-transformed proportion on log-transformed concentration and its horizontal extent covers

the range of concentrations observed in our sample. Logit(Proportion) = -7.517 + 0.902 $\log_e$(Concentration).

Fig. 1. Exceedance (negative cumulative) distributions (stepped lines) of the concentration of Pb (μg kg^{-1} d.w.) in samples of (a) liver (n = 187); and (b) bone from the femur (n = 125) of Eurasian buzzards. The curves show fitted log-normal distributions. The long-dashed vertical lines show concentrations considered to result from abnormally high exposure (a) or elevated levels (b) and the short-dashed lines denote acute exposure and absorption (a) or compatibility with lethal poisoning (b) (see text).

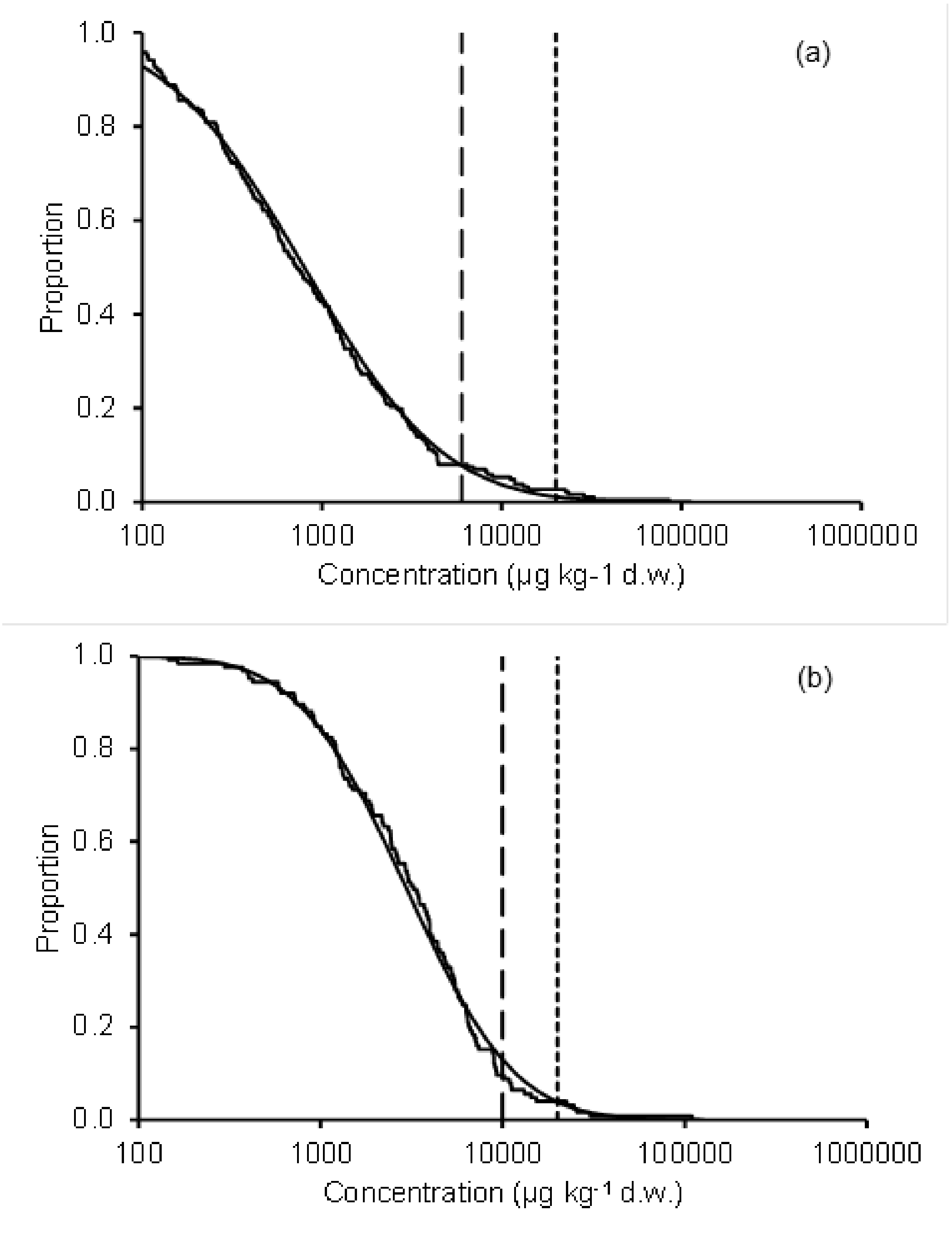


Fig. 2. Concentration of Pb (μg kg^{-1} d.w.) in samples of bone from the femur in relation to that in the liver for 92 Eurasian buzzards. The solid line shows the reduced major axis regression $\log_e(\text{Femur}) = 2.924 + 0.753 \log_e(\text{Liver})$.

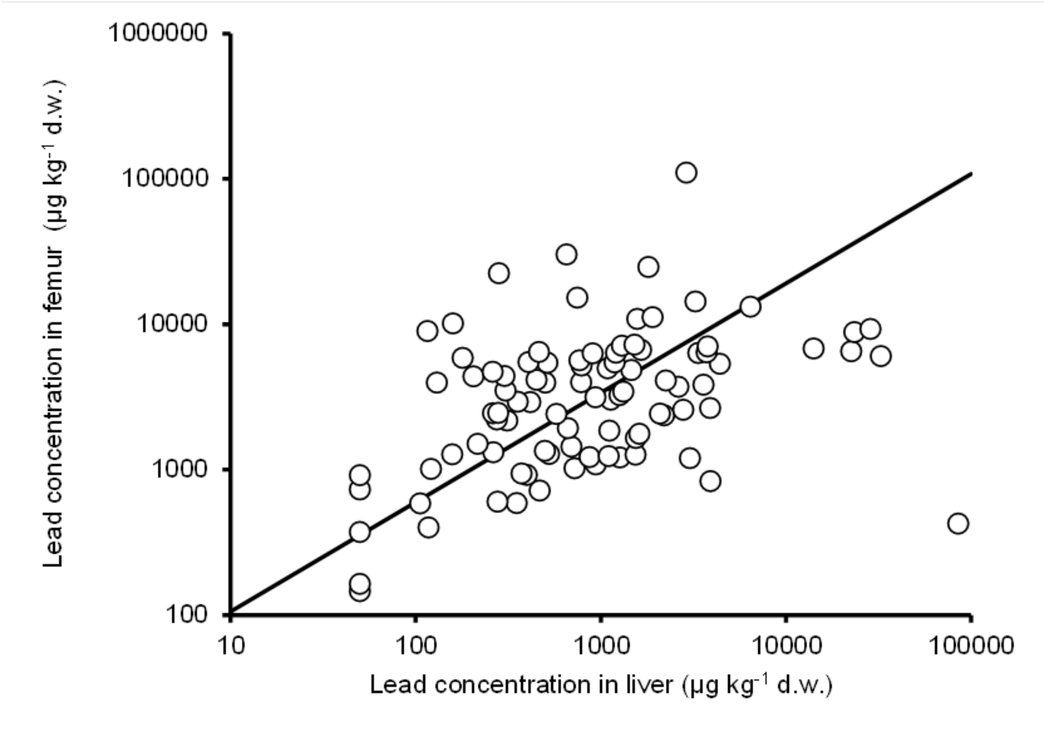


Fig. 3. Concentration of Pb in the liver for Eurasian buzzards in the UK in 2007 - 2018 in relation to collection date. Each symbol represents a determination from one individual. Modelled values (curve) are from the model with the lowest AIC_c (Model 6) of the set of models presented in Table 1. This model includes a piecewise regression effect of collection date and a sinusoidal effect of time of year, with peaks in February and troughs in August. Results for young collected in the calendar year of hatching (triangles) and older birds (circles) are distinguished, but there was no significant effect of age class on Pb concentration in the liver. Vertical grey lines show calendar years.

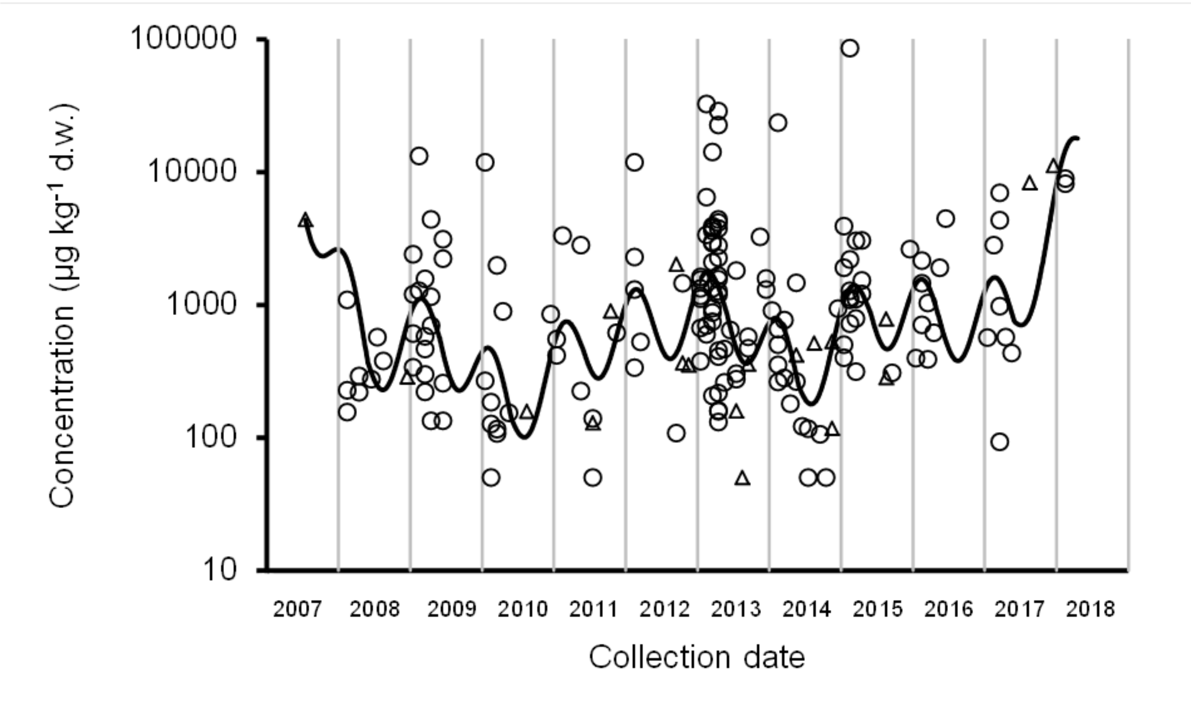


Fig. 4. Isotope ratio biplot for Pb shotgun pellets from five manufacturers; grey square Gamebore; black circle = RC, white circle = Eley; grey triangle = Lyalvale; black diamond = Hull. Each point represents a value for pellets from a single box of cartridges. The $^{208}Pb/^{206}Pb$ ratio is plotted against the $^{206}Pb/^{207}Pb$ ratio. The bivariate normal ellipse containing 95% of the modelled probability is shown.

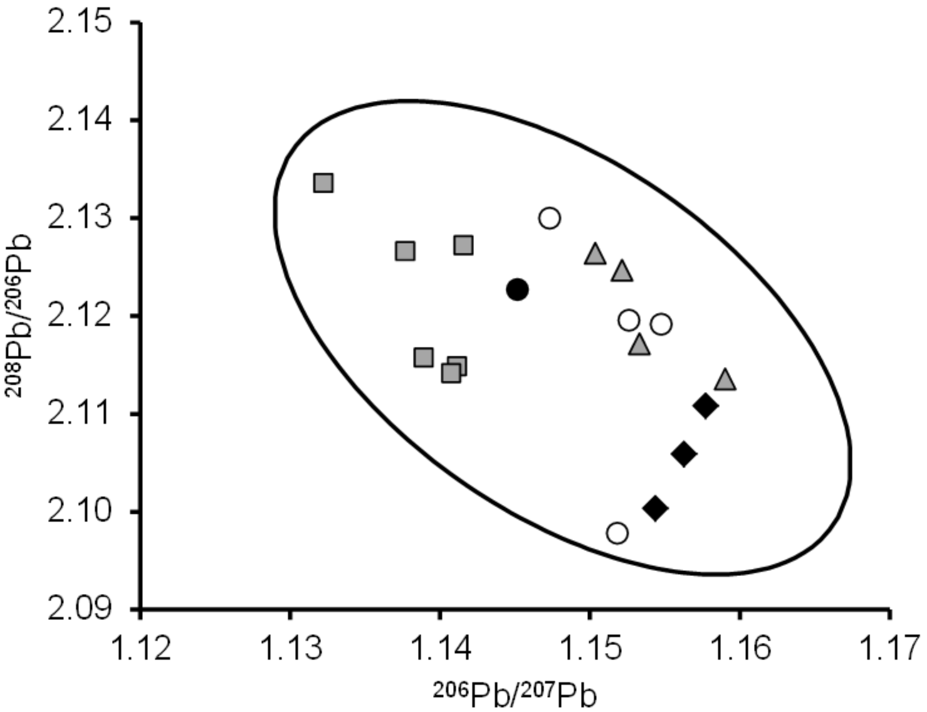
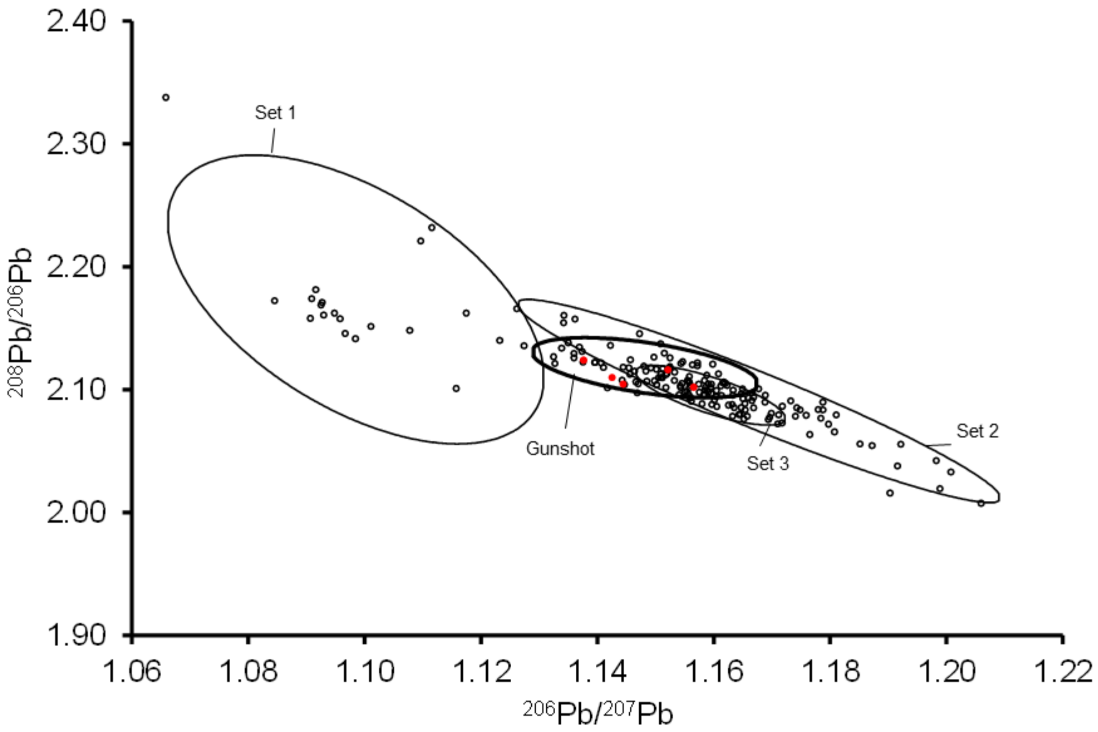


Fig. 5. Ellipses containing 95% of the probability from a bivariate normal model of isotope ratios in liver samples from Eurasian buzzards. The ellipse fitted to data for Pb shotgun pellets from cartridge brands widely used in the UK is shown by the thick line and is the same as that in Figure 4. The model also identified three additional sets with ellipses labelled Sets 1-3 and shown by the thin lines. The points represent values for individual buzzards. Individuals with liver Pb concentrations indicative of acute exposure and absorption (>20000 μg kg-1 d.w.) are shown as red circles.



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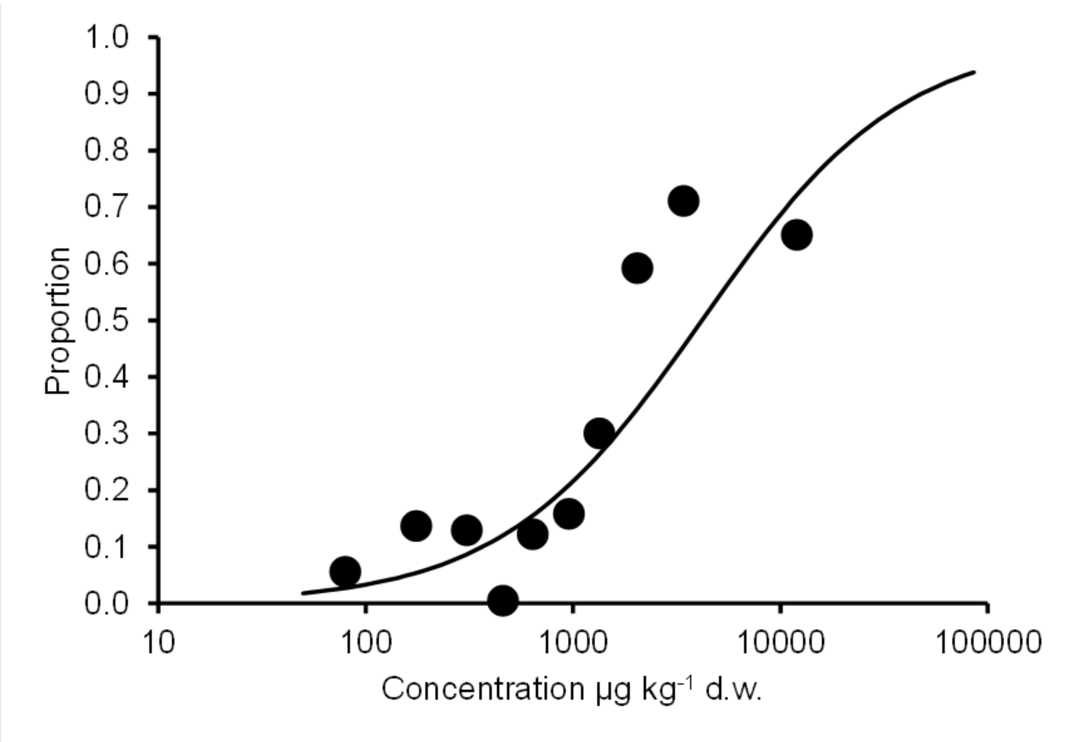

CRediT authorship contribution statement

Mark A. Taggart, Richard F. Shore: Conceptualisation, Methodology, Data curation, Chemical analysis, Writing – review. Deborah J. Pain: Conceptualisation, Methodology, Writing – review. Mónica Martinez-Haro, Rafael Mateo: Methodology, Data curation, Chemical analysis, Writing – review. Gabriela Peniche, Jemima Parry-Jones: Resources, Writing – review. Alan J. Lawlor, Elaine D. Potter, Lee A. Walker, David W. Braidwood, Andrew S. French: Methodology, Data curation, Chemical analysis. Julia Homann, Andrea Raab, Joerg Feldmann: Methodology, Data curation, Isotope analysis, Writing – review. John A. Swift: Methodology, Resources. Rhys E. Green: Conceptualisation, Methodology, Formal analysis, Writing – review.

SUPPLEMENTARY ONLINE MATERIALS

Concentration and origin of lead (Pb) in liver and bone of Eurasian buzzards (*Buteo buteo*) in the United Kingdom

Contents

Determination of Pb concentrations in livers and bone

Buzzard bone samples were dried to constant weight at 105°C and then microwave digested using concentrated nitric acid and hydrogen peroxide (both TraceMetal Grade; Fisher Scientific, UK). ~0.3g of bone (weighed to +/- 0.00001g) was placed into a digestion vessel and 2 ml of nitric acid (HNO_3) added. Vessels were then left overnight to pre-digest at room temperature. Following pre-digestion, 1 ml of hydrogen peroxide (H_2O_2) was added to each sample before microwave digestion. Digests were poured into 14ml PP (polypropylene) sample tubes; digest vessels were then rinsed (using Milli-Q) several times, adding each rinse to the tube and making up to a final volume of 10ml with Milli-Q. Pb determination in bone was achieved at the Instituto de Investigación en Recursos Cinegéticos (IREC, Cuidad Real, Spain), using graphite furnace-atomic absorption spectrometry (AAnalyst 800; Perkin-Elmer); bone meal CRM (NIST-1486) Pb recovery averaged 98% (± 8%RSD; n = 15).

Liver samples were digested and analysed at two laboratories, with the majority (*n* = 122) analysed by inductively coupled plasma-optical emission spectrometry (ICP-OES) (Varian 720-ES; Agilent) at the Environmental Research Institute (ERI, Thurso, UK) and the remainder (*n* = 65) analysed at the Centre for Ecology & Hydrology (CEH, Lancaster, UK) by inductively coupled plasma-mass spectrometry (ICP-MS) (DRCII ICPMS; Perkin Elmer). Liver samples tested at ERI were digested and prepared as for bones, while at CEH digests were undertaken using fresh tissue (~1g), HNO_3 only (10ml of 70% ultrapure (Baker, Ultrex II)) and microwave digestion. Dry weight concentrations were then recalculated based upon the wet weight of the analysed sample and the moisture content of a sub-sample. Soft tissue certified reference materials tested alongside liver samples at ERI and CEH (bovine liver BCR-185R, lobster hepatopancreas NRC-CNRC TORT-2 and dogfish liver NRC-CNRC DOLT-4) provided Pb recovery data between 89 – 107% across the various batches of samples. The limit of detection (LOD) applied here (based on procedural blank data from ICP-OES analysis of liver samples at ERI) was <100 μg kg^{-1} (in dry liver tissue). All concentrations here are expressed as μg kg^{-1} dry weight rather than as wet weight. Dry weight values are more reliable, comparable and consistent, given the effects of variation among samples in the proportion of water lost from tissues in the field post mortem and during specimen storage and preparation (Adrian & Stevens, 1979).

Isotope analysis of Pb shot pellets from shotgun cartridges and Pb in buzzard liver samples

Digests of liver tissue samples and Pb shot from ammunition cartridges were subject to Pb isotope analysis. Liver tissue digests were generated as described above, while Pb shot were simply digested at room temperature using concentrated nitric acid (TraceMetal Grade; Fisher Scientific, UK), which produced water soluble $Pb(NO_3)_2$. For each cartridge sample tested, the cartridges were opened and the Pb shot were removed. Three shotgun pellets, selected at random, were digested together. These were allowed to dissolve for >1 week in 5ml of concentrated nitric acid, after which, solutions were diluted to 50ml total volume with Milli-Q water. For isotope analysis, further dilution was required to bring levels down to a suitably low concentration for analysis.

Pb isotopes were determined in digests of liver tissue and Pb shot using ICPMS analysis, with 10 replicate readings taken per sample. The CRM NIST 981 Pb solution (certified for Pb isotopes; with Pb 206: 24.1442 ± 0.0057%, Pb 207: 22.0833 ± 0.0027%, Pb 208: 52.3470 ± 0.0086%) was used as a standard to correct for Pb isotope mass bias. Digest solutions were either directly measured or (when Pb levels were >10 μg L^{-1}) further diluted to <10 μg L^{-1} using diluted nitric acid, in order to avoid a mass bias shift within the isotope ratio measurements. Samples were measured using a standard bracketing approach, with standards used at the concentration levels expected of the samples. Isotope ratios were calculated using standard bracketing, using the standards tested before and after the samples, to calculate the mass bias for each isotope. The determined mass bias correction factor was then applied to the results of the sample.

Because an objective of our analysis of Pb isotope ratios was to assess the contribution of Pb derived from lead pellets from shotgun cartridges to the Pb found in buzzard tissues we measured Pb isotope ratios for liver, but not for bone. That is because exposure to dietary Pb from ammunition is episodic and we expected that variation among dead individuals in Pb concentration and isotope composition would be much greater for liver than for bone. This variation would therefore provide clues about short-term exposure to different Pb sources.

Comparison of the concentration of Pb in bone samples from the femur and humerus of the same individual

Measurements of the concentration of Pb in bone were available from both the femur and humerus of the same individual for seven buzzards (Fig. S2). Natural logarithms of Pb concentrations in samples of the two types of bone showed a strong and significant positive correlation (Pearson correlation coefficient, r = 0.967, P = 0.004). The RMA regression slope of the natural logarithm of humerus concentration on the natural logarithm of femur concentration was very close to 1 (1.008), which indicates that the concentrations in the two types of bone were approximately directly proportional to one another. Concentrations in the two bone types were also very similar to each other in all seven individuals and did not differ significantly (matched-pairs t-test on $\log_e$-transformed concentrations, t_6= 1.05, P = 0.335). Given this consistency in concentration across individuals between the two bone types, which has also been reported for analyses of femur and humerus Pb concentrations for Eurasian buzzards collected in Spain (Mateo et al. 2003), we concluded that the concentration of Pb in the femur was likely to be a reliable indicator of overall bone Pb levels and used determinations of Pb from the femur alone in all further analyses.

Statistical analysis of Pb isotope ratios

We performed our analysis of Pb isotope ratios as a sequence of three logical steps. Step 1 was to characterise the isotope ratios of Pb pellets from shotgun cartridges of brands widely used in the UK. We did this by fitting a least-squares bivariate normal model to the 18 values for the $^{206}Pb/^{207}Pb$ and $^{208}Pb/^{206}Pb$ isotope ratios for pellets from widely-used shotgun cartridge brands. This model has five parameters: the means and standard deviations of the $^{206}Pb/^{207}Pb$ and $^{208}Pb/^{206}Pb$ isotope ratios and the Pearson correlation r between the two ratios. For graphical presentation of the results, we used these parameter estimates to calculate the values for the edges of the ellipse that included 95% of the modelled probability.

Step 2 of our analysis was to characterise the $^{206}Pb/^{207}Pb$ and $^{208}Pb/^{206}Pb$ isotope ratios for Pb from buzzard liver samples. To do this we fitted a statistical model by maximum-likelihood to the liver sample ratios in which we assumed that the data from different individuals resulted from a mixture of several different, but potentially overlapping, bivariate normal distributions. We assumed that one of these distributions was defined by the parameters for the shotgun pellets samples estimated in Step 1. We then fitted models with between one and five additional bivariate normal distributions defined by parameters estimated from the data. We call these distributions *additional sets*. The maximum-likelihood modelling procedure (Kalbfleisch 1985) estimated the five parameters that define each bivariate normal distribution (see Step 1) for each additional set and also the proportion of the data belonging to each set. Hence, six extra parameters were estimated for each additional set included in the model. We calculated the small-sample Akaike Information Criterion (AIC_c) and AIC_c weights for each of the models with different assumed numbers of additional sets (Burnham & Anderson 2002) and selected the model with the lowest AIC_c value to use in the next step of our analysis.

Step 3 of our analysis was to assess the extent to which the probabilities of liver samples being members of the shotgun set and the additional sets were correlated with the concentration of Pb in the liver sample. This analysis was performed using the three additional sets identified by the AIC_c analysis in Step 2 (see Results). We adapted the maximum-likelihood model described for Step 2 to use the values for the means and standard deviations of $^{206}Pb/^{207}Pb$ and $^{208}Pb/^{206}Pb$ isotope ratios and the Pearson correlation r between the two ratios of the shotgun set from Step 1 and these parameters for the three additional sets estimated in Step 2. These values were treated as fixed and the model was now used to estimate only the proportions of the data belonging to each set. This was done for ten subsets of the data which were defined according to the concentration of Pb in the liver. We divided the liver samples into deciles (tenths of the distribution, each including in each decile 18 or 19 of the 182 data values) using their ranked Pb concentrations. The cut-point values separating the deciles, in rank order, were 132, 240, 380, 550, 770, 1155, 1570, 2795, and 4382 μg kg^{-1}. We estimated the proportions of data in each decile subset attributable to the shotgun set and the three additional sets and then calculated Pearson correlation coefficients and ordinary least squares regressions for the relationships, across the deciles, between the logit-transformed estimate of the proportion of the data in the shotgun set and each of the three additional sets (as the dependent variable) and the mean of the $\log_e$-transfomed Pb concentrations for samples included in each decile.

Supplementary References

Adrian, W.J. & Stevens, M.L. 1979. Wet versus dry weights for heavy metal toxicity determinations in duck liver. Journal of Wildlife Diseases, 15, 125-126.

Burnham K. P., Anderson D. R. (2002) Model selection and multi-model inference: A practical information-theoretic approach. Springer-Verlag, Berlin.

Kalbfleisch, J.G. 1985. Probability and Statistical Inference II. Springer-Verlag, New York.

Mateo, R., Taggart, M., Meharg, A.A. 2003. Pb and arsenic in bones of birds of prey from Spain. Environmental Pollution 126, 107–114.

Table S1. Details of shotgun cartridges obtained for the determination of Pb isotope ratios of shotgun pellets.

Manufacturer	Brand	Shot size (#)	Load weight (g)	Cartridge length (mm)	Date of Purchase	$^{206}Pb/^{207}Pb$	$^{208}Pb/^{206}Pb$
Eley	Grand Prix	6	30	65	21/09/2018	1.155	2.119
Eley	VIP	6	28	65	21/09/2018	1.152	2.098
Eley	VIP Game	6	30	65	19/09/2018	1.153	2.120
Eley	VIP Game	6	30	65	21/09/2018	1.147	2.130
Gamebore	Black Game	6	30	70	21/09/2018	1.132	2.134
Gamebore	Super Game	6	28	65	21/09/2018	1.141	2.115
Gamebore	Super Game	6	30	65	21/09/2018	1.142	2.127
Gamebore	Super High Bird	6	30	65	01/03/2017	1.139	2.116
Gamebore	Super High Bird	6	30	65	01/03/2017	1.141	2.114
Gamebore	Velocity	6	30	70	21/09/2018	1.138	2.127
Hull	High Pheasant	6	30	65	21/09/2018	1.156	2.106
Hull	High Pheasant	6	30	65	21/09/2018	1.158	2.111
Hull	Imperial Game	5	28	65	21/09/2018	1.154	2.100
Lyalvale Express	Special Game	6	30	65	19/09/2018	1.152	2.125
Lyalvale Express	Supreme Game	6	30	65	21/09/2018	1.159	2.114
Lyalvale Express	Supreme Game	5	32	65	21/09/2018	1.153	2.117
Lyalvale Express	Supreme Game	6	30	65	21/09/2018	1.150	2.126
RC (Italy)	Professional Game	6	30	65	21/09/2018	1.145	2.123

Table S2. Comparison of the performance of models of the Pb isotope ratios in samples of liver (*n* = 181) of Eurasian buzzards in the UK. All models included the bivariate normal model fitted to isotope ratio data for widely-used Pb shotgun pellets from five manufacturers (see Table S1). The models differed according to the number of additional sets included of subpopulations, each with its own bivariate normal distribution of isotope ratios. For each model, the number of fitted parameters, ΔAIC_c (the difference in AIC_c between the model and that with the lowest AIC_c of the set) and the AIC_c weight are given. The model with the lowest AIC_c is shown in bold.

Number of additional sets	Number of fitted parameters	ΔAIC_c	AIC_c wt
1	6	151.57	<0.001
2	12	8.37	0.012
3	**18**	**0.00**	**0.756**
4	24	2.37	0.231
5	30	14.49	0.001

LEGENDS TO SUPPLEMENTARY FIGURES

Fig. S1. Map of Britain and Ireland showing the collection localities of Eurasian buzzards for which the concentration of Pb was determined in the liver only (n = 95; triangles), femur only (n = 33; squares) or from both tissues (n = 91; circles). The collection locality of one of the specimens was uncertain and cannot be plotted.

Fig. S2. Concentrations of Pb (μg kg^{-1} d.w.) in samples of bone from the humerus and femur of the same individual for seven Eurasian buzzards. The line shows the expected relationship if concentrations were equal in the two types of bone.

Fig. S3. Concentration of Pb in the femur for Eurasian buzzards in the UK in 2008 - 2015 in relation to collection date. Each symbol represents a determination from one individual. No modelled effects of date of collection or annual cycle are shown because neither was included in the model with the lowest AIC_c (Model 1) of the set of models presented in Table 1. Results for young in the calendar year of hatching (triangles) and older birds (circles) are distinguished. Model 1 only includes the effect of age class on Pb concentration in the femur, with the concentration for young (of the year) being lower than for older birds. Vertical grey lines show calendar years.

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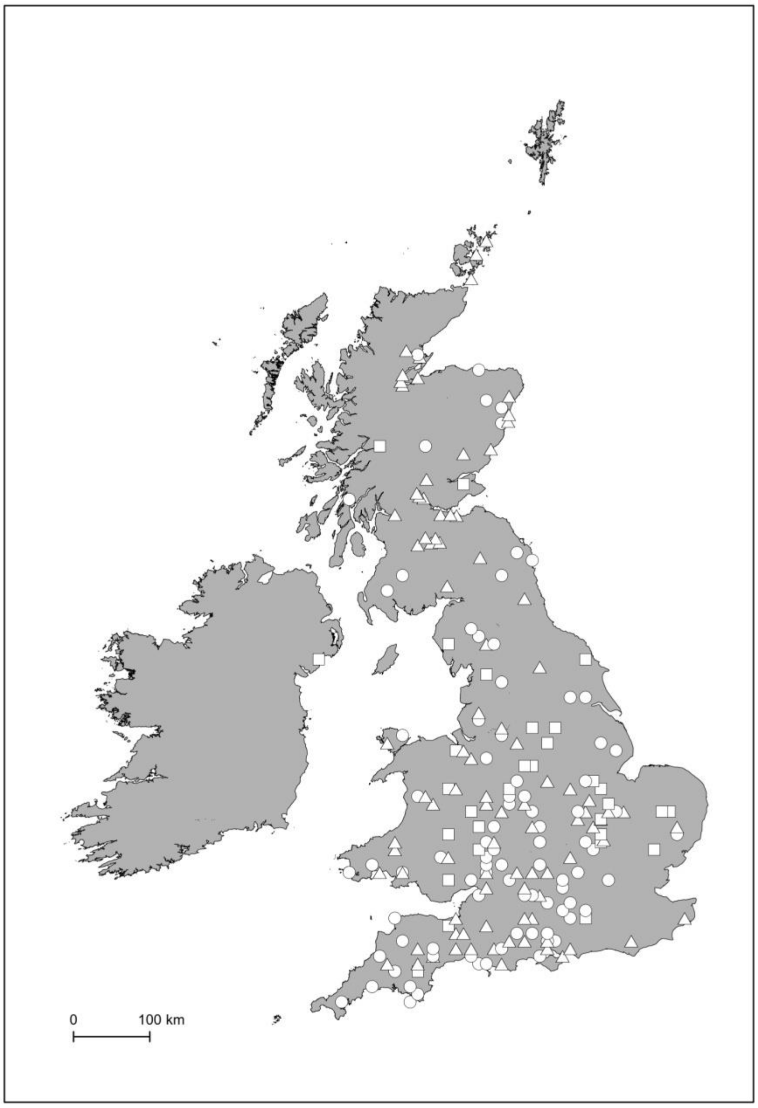

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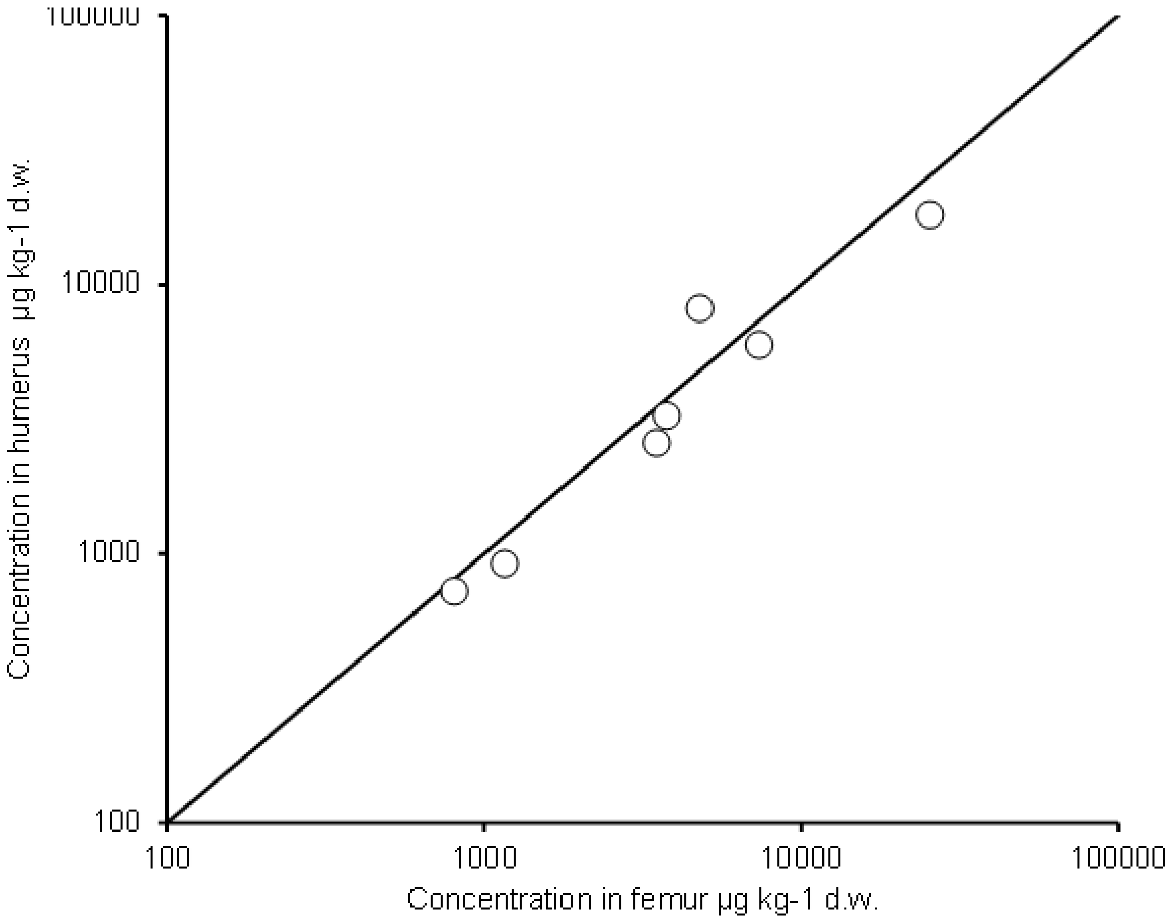

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